


PHYLOGENY OF *FOMITOPSIS PINICOLA*: A SPECIES COMPLEX


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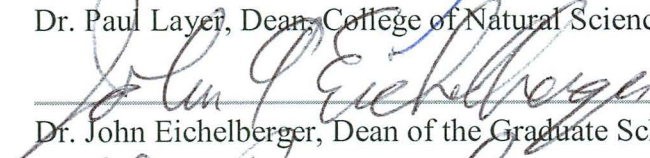


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PHYLOGENY OF *FOMITOPSIS PINICOLA*: A SPECIES COMPLEX

A

THESIS

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Abstract

Fomitopsis pinicola sensu lato is a common, saprotrophic fungal species occurring over the large circumpolar region covering Eurasia and North America, and extending south along areas of high elevation. It colonizes hardwood and softwood substrates, causing brown rot decay of wood, and exhibits a wide variety of morphological types and colors. Since the late nineteenth century, attempts have been made to determine how many species were in the genus *Fomitopsis*. Early attempts described two to three species based on host specificity and morphology. By the mid twentieth century, extensive mating studies among single spore isolates had been completed revealing three intersterility groups: two in North America and one in Europe. Partial fertility of both North American groups with the European group led researchers to describe *F. pinicola* as one biological species within which there was variation in host specificity and morphology. Further research was required using powerful molecular techniques to determine if a cryptic species complex was indeed present.

This study was undertaken to delimit phylogenetic species in *Fomitopsis pinicola sensu lato* using phylogenetic and population genetic methods. Specimens of *F. pinicola* were collected throughout its range and gene sequences were obtained for three nuclear genes: EF1A, ITS and RPB2. Concatenated, partitioned Bayesian analyses were performed and gene tree topologies were compared to identify common clades with high posterior probability support. In addition to phylogenetic methods, coalescent analyses were performed to develop a coalescent species tree, and DNA sequence polymorphism was evaluated to determine several measures of genetic variation for comparison among the

clades. These data confirmed results of the historic mating studies and supported the existence of four phylogenetic species in a species complex: *Fomitopsis pinicola* restricted to Eurasia, *Fomitopsis ochracea* restricted to North America, and two previously undescribed North American species of *Fomitopsis*.

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Phylogeny of *Fomitopsis pinicola*: A Species Complex

Abstract: Fungal species with a broad distribution may exhibit considerable genetic variation over their geographic ranges. This variation may develop among populations based on geographic isolation, lack of migration and genetic drift over time, though this genetic variation may not always be evident when examining phenotypic characters.

Fomitopsis pinicola is an abundant saprotrophic fungus found on decaying logs throughout temperate regions of the Northern Hemisphere. Phylogenetic studies have addressed the relationship of *F. pinicola* to other wood-rotting fungi, but species boundaries within *F. pinicola* have not been addressed using molecular data. While forms found growing on hardwood and softwood hosts exhibit variation in habit and appearance, it remains to be determined whether these forms are genetically distinct. In this study, we¹ generated DNA sequences of the Internal Transcribed Spacer (ITS), Elongation Factor 1A (EF1A), and RNA polymerase II subunit (RPB2) from 220 collections from across all major geographic regions where this fungus occurs, with a primary focus on North America. We utilized Bayesian and maximum likelihood analyses and evaluated the gene trees within the species tree using coalescent methods to elucidate evolutionarily independent lineages. We find that *F. pinicola sensu lato* encompasses four well-supported, congruent clades: a European clade, a southwestern U.S. clade, and two sympatric northern North American clades. Each of these clades represents distinct species according to phylogenetic and population-genetic species

¹ Haight JE, Laursen GA, Glaeser J, Taylor DL. Phylogeny of *Fomitopsis pinicola*: a species complex. In preparation for *Mycologia*.

concepts. Morphological data currently available for *F. pinicola* do not delimit these clades. *Fomitopsis pinicola*, described originally from Europe, appears to be restricted to Eurasia. Based on DNA data obtained from an isotype, one well-defined and widespread clade found only in North America represents the recently described *Fomitopsis ochracea*. The remaining two North American clades represent previously undescribed species. The amount of diversity shown in North America is quite interesting given that *F. pinicola* is thought of as a host-generalist saprotropic fungus with a broad distribution.

Key words: Bayesian, maximum likelihood, phylogenetic species

INTRODUCTION

Fomitopsis pinicola (Sw.) P. Karst. is a common brown rot fungus inhabiting softwood and hardwood substrates throughout North America, Europe and Asia. It is a major decomposer of coniferous wood throughout its range, but it is also known to colonize live trees. For example, a survey taken during logging operations of Sitka spruce, western hemlock and western red cedar in Southeast Alaska found that *Fomitopsis pinicola* was responsible for 73% of the loss due to decay in Sitka spruce and 22% in Western hemlock (Kimmey 1956). At its southern geographic extreme, it is common in declining fir forests south of Mexico City (Tovar and Garza 2007). The fungus is also an important cause of trunk rot in old growth western conifers in North America (Sinclair et al. 1987, Glaeser et al. 2009). Early mating work on this widely recognized fungus suggested reproductive isolation between some sets of isolates (Mounce and Macrae 1938), but this work has yet to be followed up with molecular analyses.

The genus Fomitopsis—The genus *Fomitopsis* is classified in the polyporoid clade within the homobasidiomycetes. Although the polyporoid clade as a whole is only weakly supported phylogenetically, the *Fomitopsis-Daedalea-Piptoporus* group, exhibiting brown rot decay and bipolar mating, is strongly supported (Hibbett and Thorn 2001). The genus *Fomitopsis* P. Karst was originally described as having a corky or woody sporocarp, the interior of which is floccose and is covered with a more or less thick, blackish crust (Karsten 1881). Karsten (1899) classified 3 species in *Fomitopsis*, namely *F. unguolata* (Schaeff.) P. Karst. (= *F. pinicola*), *F. rosea* (Alb. & Schw.) P. Karst., and *F. obducens* (Pers) P. Karst. Karsten (1899) noted that *Fomitopsis unguolata* and *F. rosea*

lacked cystidia and that the young portions of fruiting bodies had a smooth surface. He separated them by the color of their pore surface, yellowish and pink, respectively.

Fomitopsis obducens was distinguished by the presence of cystidia and some type of pubescence on young portions of fruiting bodies (Karsten 1899). Gilbertson and Ryvarden (1986) defined *Fomitopsis* as comprised of brown-rot fungi with tough perennial or rarely annual basidiocarps, with a white, tan or pinkish pore surface of small, regular pores. The hyphal system was dimitic or trimitic and clamp connections were formed in generative hyphae. They noted that basidiospores were hyaline, smooth and negative in Melzers reagent. Species included *F. cajanderi*, *F. feei*, *F. rosea*, *F.*

officinalis, *F. pinicola*, *F. spraguei*, *F. durescens*, *F. nivosus*, *F. palustris*, *F. meliae*.

While attempts have been made to narrow the genus based on micromorphological characters (Kotlaba and Pouzar 1998), molecular evidence seems to indicate that at least seven species fall within a *Fomitopsis* core group anchored by *F. pinicola* (Kim et al. 2005). Recent evidence (Kim et al. 2005) suggests that the genus *Fomitopsis* may not be monophyletic, and it may include at least one species of *Piptoporus*.

Taxonomy of Fomitopsis pinicola—The phenotypic appearance of *F. pinicola* can be quite variable with respect to color and form. It has also been collected on a variety of host species. This has led to speculation that cryptic species may be present. Fries (1821) described two species, *Polyporus marginata* being lighter in color and ungluate, and *Polyporus pinicola*, with a cap color tending to black and cinnamon. The host range of *P. marginata* included *Fagus*, *Betula*, *Pinus* and *Pyrus*, while that of *P. pinicola* included *Abies*, and *Betula*. Saccardo (1888) described a third species, *Fomes ungluates*, having a

cap with thick, concentric reddish-ochre colored furrows, which was collected on conifers in the Italian alps. Mounce (1929) found various forms and colors of sporocarps on *Tsuga*. Her extensive collections and work with crosses of single spore isolates lead her to agree with Hedgecock (1914), Lloyd (1915), Murrill (1908) and Overholts (1915) that the three species described by Saccardo were forms of the same species. She discovered that monosporous mycelia of *F. pinicola* isolated from sporophores collected on deciduous hosts were mutually fertile with monosporous mycelia isolated from sporophores from coniferous hosts. She concluded that *F. marginalis* and *F. pinicola* were the same species and that European and American forms of the fungus were identical to each other. Further work with monosporus pairings lead to the discovery of two intersterile populations in North America (Mounce and Macrae 1938). Describing them as separate species was rejected when both populations were found to form fertile spores when monosporous cultures of each were crossed with those from Europe. Though inhabiting a large geographic range, European populations of *F. pinicola* were found to be members of one intersterility group (Högberg et al. 1999).

A recently described species, *F. ochracea*, originally collected in Alberta, Canada on *Populus tremuloides* (Ryvarden and Stokland 2008), was delimited from *F. pinicola* based largely on pore color and spore morphology. Ryvarden and Stokland (2008) reported that the pore surface of *F. ochracea* did not exhibit a change from cream to citrus yellow when bruised, as seen in *F. pinicola*, and that the basidiospores were globose to broadly ellipsoid, rather than cylindrical. They noted that the smooth ochraceous pileus of *F. ochracea* is easily separated from that of *F. pinicola*. They also

obtained a sequence from the holotype and compared it to sequences of several *F. pinicola* from North America, but did not make formal comparisons to other collections.

Host range—Although typically found inhabiting softwood stumps in a saprotrophic lifestyle, *F. pinicola* colonizes a broad range of host trees. Mounce (1929) compiled a list of 91 host species of both conifers and hardwoods across North America, Europe and Asia. A search of the USDA-ARS fungal database (<http://nt.ars-grin.gov/fungaldatabases/>) for *F. pinicola* and its synonyms returned 434 entries of fungus-host combinations including 219 different hosts. Common softwood hosts in North America included *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Thuja* and *Tsuga*. The fungus has also been found on *Acer*, *Alnus*, *Betula*, *Fagus*, *Populus*, and *Prunus* among North American hardwood trees.

Geographic distribution—The range of *F. pinicola* is circumpolar but also includes temperate regions in North America, Europe and Asia. In Europe, it has been documented in Norway, Sweden, Denmark and France (Spaulding 1961), Germany (Schmid-Heckel 1988), England (Legon et al. 2005), Poland (Filsiška 1997) and Russia (Hermansson 1997). In Asia it has been recorded in China (Teng 1996), Japan (Kobayashi 2007), India (Pande and Rao 1998), Taiwan (Chen 2002, Anonymous 1979), Phillipines (Quiniones 1980) and Korea (Cho and Shin 2004). In this study we used sequence data from the Internal Transcribed Spacer (ITS), Elongation Factor 1A (EF1A), RNA polymerase II subunit (RPB2), ATP6 and the mitochondrial large RNA (LrRNA) gene regions to assess whether *F. pinicola* comprises a species complex. We attempted to define species boundaries using both phylogenetic and coalescent population genetic methods.

MATERIALS AND METHODS

Sample Collections—To encompass the largest possible genetic diversity, fresh fruiting body data were augmented by data collected from herbarium specimens and cultures, which allowed us to increase the geographical span of the dataset. Sporophores were collected from decaying softwood and hardwood substrates in various habitat types including circumpolar subarctic boreal forests, coastal temperate rain forests, northern mixed hardwood forests and dry, southern coniferous forests at high elevations (TABLE 1). These hardwood and softwood substrates can be found in three forms: live trees, snags and logs. Fresh *F. pinicola* specimens were removed from woody substrates using a hammer and chisel. Samples were labeled with a field collection data card based on guidelines of the British Mycological Society (Iliffe 2006), wrapped in wax paper and stored in brown paper bags for transport to the laboratory.

Herbaria and Culture Collections—Dried specimens of *F. pinicola* were sampled from the Gary A. Laursen Herbarium, University of Washington, formerly located at the University of Alaska, Fairbanks; the Center for Forest Mycology Herbarium, US Forest Service, Madison, WI; the U.S. National Fungus Collections (BPI), Beltsville, MD; the National Herbarium Nederland, Universiteit Leiden Branch, Leiden, The Netherlands; and the Royal Ontario Museum Fungarium, Ontario, Canada. Fungal cultures were obtained from private and public culture collections including the Center for Forest Mycology Culture Collection, U.S. Forest Service, Madison, WI.

TABLE I. Collection of *Fomitopsis* species complex used in phylogenetic analysis, origin, repository, and GenBank numbers

Taxon name	Collection number	Country	State/Province/ District	Collector	Host	Herbarium code	GenBank accession numbers		
							ITS	rPB2	EF1A
<i>F. ochracea</i>	DLL-3	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169588	KF169657	KF178313
<i>F. ochracea</i>	DLL-4	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169589	KF169658	KF178314
<i>F. ochracea</i>	FP-125083-T	USA	New Hampshire	A.L. Shigo	<i>Tsuga</i>	CFMR	KF169590	KF169659	KF178315
<i>F. ochracea</i>	HHB-17611	USA	Alaska/Kenai	H.H. Burdsall	<i>Picea</i>	CFMR	KF169591	KF169660	KF178316
<i>F. ochracea</i>	HHB-19667	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169592	KF169661	KF178317
<i>F. ochracea</i>	HHB-19670	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169593	KF169662	KF178318
<i>F. ochracea</i>	HHB-19692	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169594	KF169663	KF178319
<i>F. ochracea</i>	HHB-3331-Sp	USA	Michigan	H.H. Burdsall	<i>Acer</i>	CFMR	KF169595	KF169664	KF178320
<i>F. ochracea</i>	JEH-12C	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169596	KF169665	KF178321
<i>F. ochracea</i>	JEH-12E	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169597	KF169666	KF178322
<i>F. ochracea</i>	JEH-12F	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169598	KF169667	KF178323
<i>F. ochracea</i>	JEH-13A	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169599	KF169668	KF178324
<i>F. ochracea</i>	JEH-13B	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169600	KF169669	KF178325
<i>F. ochracea</i>	JEH-13D	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169601	KF169670	KF178326
<i>F. ochracea</i>	JEH-37	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169602	KF169671	KF178327
<i>F. ochracea</i>	JEH-38	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169603	KF169672	KF178328
<i>F. ochracea</i>	JEH-79	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169604	KF169673	KF178329
<i>F. ochracea</i>	JEH-80-ss1	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169605	KF169674	KF178330
<i>F. ochracea</i>	JEH-81	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169606	KF169675	KF178331
<i>F. ochracea</i>	JEH-83-ss2	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169607	KF169676	KF178332
<i>F. ochracea</i>	JEH-85	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169608	KF169677	KF178333
<i>F. ochracea</i>	JEH-87-ss5	Canada	British Columbia	J.E. Haight	<i>Populus</i>	CFMR	KF169609	KF169678	KF178334
<i>F. ochracea</i>	JEH-87-ss7	Canada	British Columbia	J.E. Haight	<i>Populus</i>	CFMR	KF169610	KF169679	KF178335
<i>F. ochracea</i>	JEH-88	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169611	KF169680	KF178336
<i>F. ochracea</i>	JEH-91	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169612	KF169681	KF178337

TABLE I. continued...

Taxon name	Collection number	Country	State/Province/ District	Collector	Host	Herbarium code	GenBank accession numbers		
							ITS	rPB2	EF1A
<i>F. ochracea</i>	KTS-28	USA	Vermont	K.T. Smith	<i>Picea</i>	CFMR	KF169613	KF169682	KF178338
<i>F. ochracea</i>	LT-12	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169614	KF169683	KF178339
<i>F. ochracea</i>	LT-16	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169615	KF169684	KF178340
<i>F. ochracea</i>	LT-18	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169616	KF169685	KF178341
<i>F. ochracea</i>	LT-19	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169617	KF169686	KF178342
<i>F. ochracea</i>	LT-17	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169618	KF169687	KF178343
<i>F. ochracea</i>	48800	Canada	Newfoundland	L. Ryvardeen	<i>Picea mariana</i>	TRTC	KF169619	KF169688	KF178344
<i>F. ochracea</i>	PEL-Lk-6-1	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169620	KF169689	KF178345
<i>F. pinicola</i>	LT-323	Estonia	Tartumaa	D.L. Taylor	<i>Picea</i>	CFMR	KF169651	KF169720	KF178376
<i>F. pinicola</i>	LT-319	Estonia	Tartumaa	D.L. Taylor	<i>Picea</i>	CFMR	KF169652	KF169721	KF178377
<i>F. pinicola</i>	FCUG 2034	Sweden	Not recorded	K-H. Larsson	Not recorded	GB	KF169653	KF169722	KF178378
<i>F. pinicola</i>	FCUG 2056	Sweden	Not recorded	K-H. Larsson	Not recorded	GB	KF169654	KF169723	KF178379
<i>F. pinicola</i>	HK-19330	Russia	South Ural	H. Kotiranta	<i>Picea</i>	H	KF169655	KF169724	KF178380
<i>F. pinicola</i>	TS-Fp-24	Russia	Moscow	T. Semenova	<i>Picea</i>	MW	KF169656	KF169725	KF178381
<i>F. sp.</i>	32TT	USA	Washington	C.G. Shaw	<i>Dendroctonus</i>	CFMR	KF169621	KF169690	KF178346
<i>F. sp.</i>	CS-1	USA	Oregon	C.L. Schmitt	<i>Abies</i>	CFMR	KF169622	KF169691	KF178347
<i>F. sp.</i>	DR-301	USA	Michigan	D.L. Richter	<i>Picea</i>	CFMR	KF169623	KF169692	KF178348
<i>F. sp.</i>	DR-366	USA	Michigan	D.L. Richter	<i>Acer</i>	CFMR	KF169624	KF169693	KF178349
<i>F. sp.</i>	DR-472	USA	Michigan	D.L. Richter	<i>Populus</i>	CFMR	KF169625	KF169694	KF178350
<i>F. sp.</i>	JAG-08-19	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169626	KF169695	KF178351
<i>F. sp.</i>	JAG-08-20	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169627	KF169696	KF178352
<i>F. sp.</i>	JAG-08-25	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169628	KF169697	KF178353
<i>F. sp.</i>	JEH-78	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169629	KF169698	KF178354
<i>F. sp.</i>	JEH-82	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169630	KF169699	KF178355
<i>F. sp.</i>	JEH-86	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169631	KF169700	KF178356

TABLE I. continued...

Taxon name	Collection number	Country	State/Province/ District	Collector	Host	Herbarium code	GenBank accession numbers		
							ITS	rPB2	EF1A
<i>F. sp.</i>	JEH-146	USA	Wisconsin	A.D. Parker	<i>Larix</i>	CFMR	KF169632	KF169701	KF178357
<i>F. sp.</i>	JEH-147	USA	Wisconsin	A.D. Parker	<i>Larix</i>	CFMR	KF169633	KF169702	KF178358
<i>F. sp.</i>	KM-1	USA	Oregon	K. Mallams	<i>Abies</i>	CFMR	KF169634	KF169703	KF178359
<i>F. sp.</i>	LT-5	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169635	KF169704	KF178360
<i>F. sp.</i>	MJL-112-Sp	USA	New York	M.J. Larsen	<i>Abies</i>	CFMR	KF169636	KF169705	KF178361
<i>F. sp.</i>	FP-105760-T	USA	Idaho	R.W. Davidson	<i>Pinus</i>	CFMR	KF169637	KF169706	KF178362
<i>F. sp.</i>	FP-133890-T	USA	Montana	M.J. Larsen	Conifer	CFMR	KF169638	KF169707	KF178363
<i>F. sp.</i>	FP-125086-T	USA	New Hampshire	A.L. Shigo	<i>Tsuga</i>	CFMR	KF169639	KF169708	KF178364
<i>F. sp.</i>	JS-22	USA	Maine	J. Schilling	<i>Picea</i>	CFMR	KF169640	KF169709	KF178365
<i>F. sp.</i>	FP-105881-R	USA	Colorado	T.E. Hinds	<i>Pinus</i>	CFMR	KF169641	KF169710	KF178366
<i>F. sp.</i>	JEH-142-ss12	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169642	KF169711	KF178367
<i>F. sp.</i>	JEH-142-ss14	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169643	KF169712	KF178368
<i>F. sp.</i>	JEH-142-ss5	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169644	KF169713	KF178369
<i>F. sp.</i>	JEH-142-ss6	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169645	KF169714	KF178370
<i>F. sp.</i>	JW24-525-0-sap	USA	South Dakota	J.J. Worrall	<i>Pinus</i>	CFMR	KF169646	KF169715	KF178371
<i>F. sp.</i>	JW24-549B-1-sap	USA	South Dakota	J.J. Worrall	<i>Pinus</i>	CFMR	KF169647	KF169716	KF178372
<i>F. sp.</i>	JW18-240-1-sap	USA	Colorado	J.J. Worrall	<i>Pseudotsuga</i>	CFMR	KF169648	KF169717	KF178373
<i>F. sp.</i>	JW-F.pinicola#2	USA	Colorado	J.J. Worrall	<i>Picea</i>	CFMR	KF169649	KF169718	KF178374
<i>F. sp.</i>	RLG-10752-Sp	USA	Arizona	R.L. Gilbertson	<i>Pseudotsuga</i>	CFMR	KF169650	KF169719	KF178375

DNA Isolation—Tissue samples for DNA extraction were removed from the interior of fruiting body pilei. Tissue specimens were then either ground in a detergent solution of cetyl trimethyl ammonium bromide (CTAB) in glass tissue grinders (Kimble Chas Kontes, size 24), between frosted glass slides (VWR), or under liquid nitrogen in a porcelain mortar. Tissue samples for DNA extraction taken from cultures were removed by gently scraping hyphae from the surface of the agar plate and then grinding the sample under CTAB between two glass slides. Difficult cultures were grown in 1% malt liquid media, freeze dried, and ground under liquid nitrogen. After grinding the tissue by one of the preceding methods, the DNA samples were stored overnight at -20°C before 2-hour incubation in a water bath at 65°C . Following centrifugation at 14,000 RCF, the supernatant was drawn off and mixed with an equal amount of 100% isopropanol and incubated overnight at 0°C . The solution was centrifuged at 0°C for 30 minutes. The liquid was then drawn off and the precipitate was washed in 70% ethanol and then allowed to dry for 15 minutes before being dissolved in 50 μl of molecular grade water. The resulting DNA was cleaned using a GeneClean Kit (Bio 101, Inc., Carlsbad, CA, USA) with glass milk as described in Taylor & Bruns (1997). A subset of challenging samples were extracted using Qiagen Plant DNEasy kits (QIAGEN Sciences, Germantown, Maryland).

Target Loci—We targeted the following loci for amplification and sequencing: Internal Transcribed Spacer (ITS), Elongation Factor 1A (EF1A), RNA polymerase II subunit (RPB2), ATP synthase subunit 6 (ATP6), and the mitochondrial large RNA gene (LrRNA). The ITS primers ITS1F and ITS4 produced strong PCR amplification and

clean sequences. In contrast, AFTOL versions of the primers for RPB2 and EF1A resulted in poor amplification from *F. pinicola*, and therefore new primers were designed for EF1A, RPB2, ATP6 and mtLSU as follows. First, target regions from closely related fungi, such as other species of *Fomitopsis*, *Piptoporus*, *Trametes*, and *Ganoderma*, were downloaded from GenBank. These sequences were then used in blast searches against the draft genome of *F. pinicola* using tools on the DOE Fungal Genome Portal. The corresponding regions of the *F. pinicola* genome were extracted and aligned to those of related fungi using Muscle (Edgar 2004). The positions of standard AFTOL primers were then located on the resulting alignments. Considerable mismatches with *F. pinicola* were noted. In most cases, we were able to simply exchange mismatching bases with a matching base in *F. pinicola*, using the same primer positions, to design *Fomitopsis*-specific primers. We utilized degenerate bases in situations where there were variable positions within the genus *Fomitopsis*. Annealing temperatures and potential for self and cross-dimers were evaluated using NetPrimer (Premier Biosoft). Possible primers with dimers stronger than -8kcal/mol or NetPrimer scores below 80 were discarded. Primers utilized in this study are listed in TABLE 2.

Polymerase Chain and Sequencing Reactions—Polymerase Chain Reactions (PCR) were run using GoTaq DNA polymerase kits (Promega, Madison, WI). Reactions with a total volume of 15 μ l were set up containing 3 μ l of 5x GoTaq buffer having concentrations of 200 μ M dNTPs, 0.2 μ M forward and reverse primers, 0.375 units of *Taq* DNA polymerase and 3 μ l of sample DNA. PCR protocols were run for 36 cycles on a MJ Research PTC-200 Thermo Cycler at annealing temperatures specific for each primer

(TABLE 2). PCR products were run on a 1.8% agarose gel and stained with ethidium bromide to visualize the bands and were purified using an ExoSap-IT kit (GE Healthcare). Sequencing reactions were carried out in-house using the Applied Biosystems Big Dye v3.1 Terminator Cycle Sequencing Kits (Invitrogen) and were purified using a CleanSeq Sequence Cleanup kit (Agencourt). The in-house protocol for sequencing reactions was 96 degrees for 30s, 50 degrees for 30s, 60 degrees for three minutes, and the cycle repeated 30 times before ending at eight degrees until removed. Cycle sequence products were run on an ABI 3730xl DNA Analyzer (Life Technologies, Grand Island, NY) at the University of Wisconsin Biotechnology Center, Madison,

TABLE 2. Primer sequences and annealing temperatures used for each primer

Primer	Annealing temp. (°C)	Primer sequence
ITS1F	50	CTTGGTCATTTAGAGGAAGTAA
ITS4	50	TCCTCCGCTTATTGATATGC
Fp_rPB2_6.2F	50	CACTGGGGTATGGTCTGTCC
Fp_rPB2_7.2R	50	ATGTTCCGCCATAGTGCCAT
EF1-893F_Fp	50	TCACCGTGACTIONCATCAAGAA
EF1-1567R_Fp	50	GTCCCGATACCACCAATCTT
ML_Fp1F	53	GCATAATTCTCCGAAGAGTT
cML5_Fp	53	TATGAGGTGAACTTGCCGA
ML5_Fp	56	CTCGGCAAGTTCACCTCATAAG
ML6_Fp	56	CTACAGTAAAGCTGCATAGGGTC
ML_7	55	GACCCTATGCAGCTTTACTGTA
ML_8	55	TTATCCCTAGCGTAACTTTTATC
ATP6-3*	48	TCTCCTTTAGAACAATTTGAA
ATP6-Fp1	48	CTTCTAAATGGTCTATTGCTC
ATP-2*	48	GTAATTC AATAGCATCTTTAATATA

Wisconsin. A subset of raw PCR products was sent to Functional Biosciences, Inc., Madison, Wisconsin for sequencing.

Analysis—Raw sequences were processed using CodonCode Aligner 3.7.1.1 (CodonCode Corporation, Centerville, Massachusetts). Sequences were imported, bases were called and Phred scores were obtained based on the default settings. Forward and reverse sequences from each primer pair were then assembled automatically. End clipping was accomplished by setting a minimum threshold of a 1% error rate, corresponding to a consensus reading of 20, and terminal bases below the minimum threshold were then trimmed using the clip ends feature. The same minimum threshold was used to evaluate the rest of the sequence, though some user discretion was used in cases of a short region of low quality bases that could still easily be aligned. Processed sequences were initially aligned using CLUSTALW (Thompson et al. 1994) and then adjusted manually.

To determine the best model of nucleotide substitution for use within the tree building programs, we used the programs MrModeltest 2.3 (Nylander, J. A. A. 2004) and MODELTEST 3.7 (Posada and Crandall 1998). These programs select the model of nucleotide substitution that best fits the data. Analyses were conducted using maximum likelihood in RAxML 7.3.0 (Stamatakis 2006) and Bayesian analyses in MrBayes v. 3.2 (Hulslenbeck and Ronquist 2001).

Phylogenetic Tree Inference—Initially a pilot dataset of 30 sequences for each gene was analyzed using MrBayes to determine if the numbers of parsimony informative characters were sufficient to delimit clades within *F. pinicola*. Once the variability of a gene was

determined to be sufficient, the dataset of informative genes was expanded numerically and geographically. These larger datasets were analyzed for each gene using maximum likelihood in RAxML with 1000 rapid bootstrap replicates and Bayesian inference in MrBayes. Sequences for *Fomitopsis palustris*, *Fomitopsis meliae* and *Piptoporus betulina* were downloaded from GenBank and added to the ITS and RPB2 alignments for use as outgroups, however these sequences proved difficult to align unambiguously and produced long branches in the gene trees (data not shown). A suitable outgroup also could not be found for use with EF1A, therefore we were forced to use midpoint rooting for all 3 genes. Bayesian and likelihood analyses were initially performed for each gene region separately using all available data. Then a 1739 base pair concatenated multi-gene alignment was constructed using MEGA (Tamura et al. 2011) consisting of 70 collections for which we had sequences from all three final loci, namely RPB2, EF1A and ITS. This concatenated alignment was partitioned by gene, by codon position and non-coding region, and analyzed using MrBayes. The model for the run was nst=6, rates=invgamma and 3000000 generations.

Coalescent Species Tree Inference—Gene trees and the species tree may not have the same topology due to incomplete lineage sorting. To account for these potential differences, the multi-species coalescent tree was estimated using MrBayes v. 3.2. The 1739 base pair concatenated alignment was run using species partitions based on well-supported clades identified in the concatenated Bayesian analysis. The analysis model was run with settings as described in the program manual (Ronquist et al. 2011): set speciespartitions = species, unlink topology=(all), prset topologypr = speciestree, prset

brlenspr = clock:speciestree, prset popvarpr=variable, prset popsizepr=lognormal

(4.6,2.3), and using nst=6, rates = gamma, and run for 5000000 generations.

Bayesian phylogenetics and phylogeography—In order to test the species boundaries suggested by the coalescent analysis in MrBayes, the 70 collection, 3 gene dataset was further analyzed using the program BPP (Rannala and Yang, 2003; Yang and Rannala, 2010) in the species delimitation mode using rjMCMC algorithms. This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. A gamma prior $G(2, 1000)$, with mean $2/2000 = 0.001$, was used on the population size parameters (θ s). The age of the root in the species tree (τ_0) was assigned the gamma prior $G(2, 1000)$, while the other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala, 2010: equation 2). Choosing one of the interior nodes for collapsing generated a starting tree. Each analysis was run using both algorithm 0 and algorithm 1 (Yang and Rannala, 2010) with at least three different starting trees each to confirm consistency between runs. Posterior probabilities were further confirmed by artificially splitting a single phylogenetic clade into sister population, creating a new guide tree containing these sister species, and observing whether the artificial nodes thus created collapsed during the species delimitation runs. Upon completion of each run, a posterior probability is assigned to each node of the user supplied guide tree, which is the probability of a speciation event. A probability of ≥ 0.95 may be interpreted as a species split, since a probability of 1 means that the rjMCMC algorithm supported that node each time it was evaluated. (Leaché and Fujita 2010).

Population Genetics Parameter Estimation

Haplotype Reconstruction—Cases of ambiguous base calls were commonly found in RPB2 and EF1A gene sequences, indicating individuals heterozygous for that SNP. Haplotype estimates for the heterozygous sequences were determined using the program PHASE version 2.1 (Stephens et al. 2001; Stephens and Donnelly 2003), using SEQPHASE (Flot 2010) to develop the input files. Program default values were used for number of iterations, thinning interval and number of burn-in generations. Haplotype frequency was checked between runs for consistency. Goodness-of-fit results were checked between two runs and then the program was set to automatically run five independent runs using the `-x` option to output the haplotype estimate corresponding to the run with the best average goodness-of-fit. Due to the large number of SNPs, each clade (NAA, NAB, SW1, SW2 and EUR) was run in PHASE separately. The PHASE output files were processed in SEQPHASE and the resulting FASTA files were aligned using MEGA and saved as a NEXUS file. A haplotype tree was produced using MrBayes and analyzed for the presence of shared haplotypes between clades.

Sequence polymorphism—To measure DNA polymorphism and nucleotide divergence among the sequences in our dataset, the haplotype alignments for RPB2, EF1A and ITS were concatenated and aligned using SeAl (Rambaut), and analyzed in DnaSP (Librado and Rozas, 2009). Nucleotide divergence was measured between each pair of clades. Estimates of divergence included total number of polymorphic sites, number of fixed differences, the number of polymorphic sites present in the one clade but monomorphic in the second clade, total shared mutations between clades, average number of nucleotide

differences between clades, average number of nucleotide differences between populations (K), nucleotide divergence (D_{xy}) and the number of net nucleotide substitutions per site between populations (Da).

RESULTS

Primers—The custom designed EF1A and RPB2 primers (TABLE 2) provided improved amplification, demonstrated by the improved number and quality of PCR bands recovered and cleaner sequencing results. The custom forward primer ATP6-Fp1 performed better than ATP6-3* when combined with the reverse primer ATP-2*, generating more bands on agarose gels. The custom mtLSU primer pairs also worked well, generating PCR products with strong bands, of which the primer pair ML_Fp1F and cML5Fp returned the best results. The EF1A and RPB2 genes displayed considerable intra-specific variability in *F. pinicola*. Out of 557 total characters in the EF1A alignment, 32 were parsimony informative. The RPB2 gene sequence yielded an alignment 638 bases, of which 37 positions were parsimony informative. The ITS alignment was 544 characters with only 14 parsimony informative characters. A 24-sample subset of the genomic DNA amplified with primers ATP6-Fp1 and ATP6-2* yielded an aligned dataset with no variability. Likewise, a 24-sample subset of the genomic DNA amplified with primer sets ML_5Fp1 and cML5_Fp, ML5_Fp and ML6_Fp, and ML_7 and ML_8 also yielded an aligned dataset with no variation. Therefore the ATP-6 and mtLSU genes were dropped from further consideration in this study.

DNA extraction, amplification and sequencing—A total of 194 samples of genomic DNA were extracted representing the known habitat range of *F. pinicola*. We obtained 177 passing ITS sequences, which, along with additions downloaded from GenBank, resulted in 220 ITS sequences. Our sequences plus additions from GenBank yielded 132 EF1A

sequences and 112 RPB2 sequences. In total, 70 collections were sequenced in common over the 3 gene regions (TABLE 1).

Gene trees—Gene trees for RBB2, EF1A and ITS each supported the presence of four to five major clades within our collections of *Fomitopsis pinicola* (FIGS. 1-3). Two clades, North American Clades A and B (NAA and NAB), occurred in an area of North America ranging from Interior Alaska to Maine, two clades (Southwest Clades 1 and 2) occurred in an area ranging from Arizona to western South Dakota, and one clade (European Clade) was specific to Europe and Asia. Individuals from NAA and NAB were found to inhabit the same range and occasionally the same site. Members of NAA and NAB colonized hardwoods and softwoods. Members of Southwest Clades 1 and 2 (SW1 and SW2) were only collected on conifers. Members of the European Clade represent specimens ranging from Sweden to Siberia and were collected on both hardwoods and softwoods. There were members found in every clade that exhibited context colors commonly described in *F. pinicola*; cream, grey, red, brown and black.

The tree topologies resulting from Bayesian analysis for RPB2, EF1A and ITS were similar, although posterior probabilities (pp) varied among the branches. The RPB2 tree (FIG. 1) shows NAA with strong separation (pp 1.0) from the other clades. The European Clade is represented, but split into two clades (pp 0.98 for each) within a mixed group of SW1, SW2, and NAB. The EF1A tree (FIG. 2) showed a basal split of NAB, EUR, SW1 and SW2 from NAA (pp 1.0). While clades NAB, SW1 and SW2 had low support, support for the Eur clade was significant (pp 0.99). The ITS tree (FIG. 3) supported clade NAB at pp 0.61. Within that clade was a branch (pp 0.95) within which fell SW1 (pp

0.99) and SW2 (pp 1.0) and a few other collections from Arizona, New Mexico and Colorado. The Eur clade was supported at 1.0 pp, with a few European collections that fell near, but not within the clade. Clade NAA was on a separate branch, but also with low support (0.61 pp).

Species tree—The coalescent species analysis run in MrBayes produced a tree of five clades (FIG. 4) with posterior probabilities of 100%: NAA, NAB, SW1, SW2, and Eur. Clade SW2 was represented by 2 individuals in the coalescent species tree. The tree had a similar topology to the three gene trees, but the coalescent provided a more distinct branching pattern and better definition for the boundary of each clade. The most basal node still separated NAA from the rest of the clades, but the European Clade now was distinctly separated from NAB and SW.

Testing species boundaries—We ran algorithm 0 and algorithm 1 in BPP on a five species guide tree [(((Eur, NA2), (SW1, SW2)), NA1)], which produced results supporting the five populations diagnosed in the coalescent species tree. Each of the five nodes had a posterior probability of 100%. Artificially splitting North American clade A

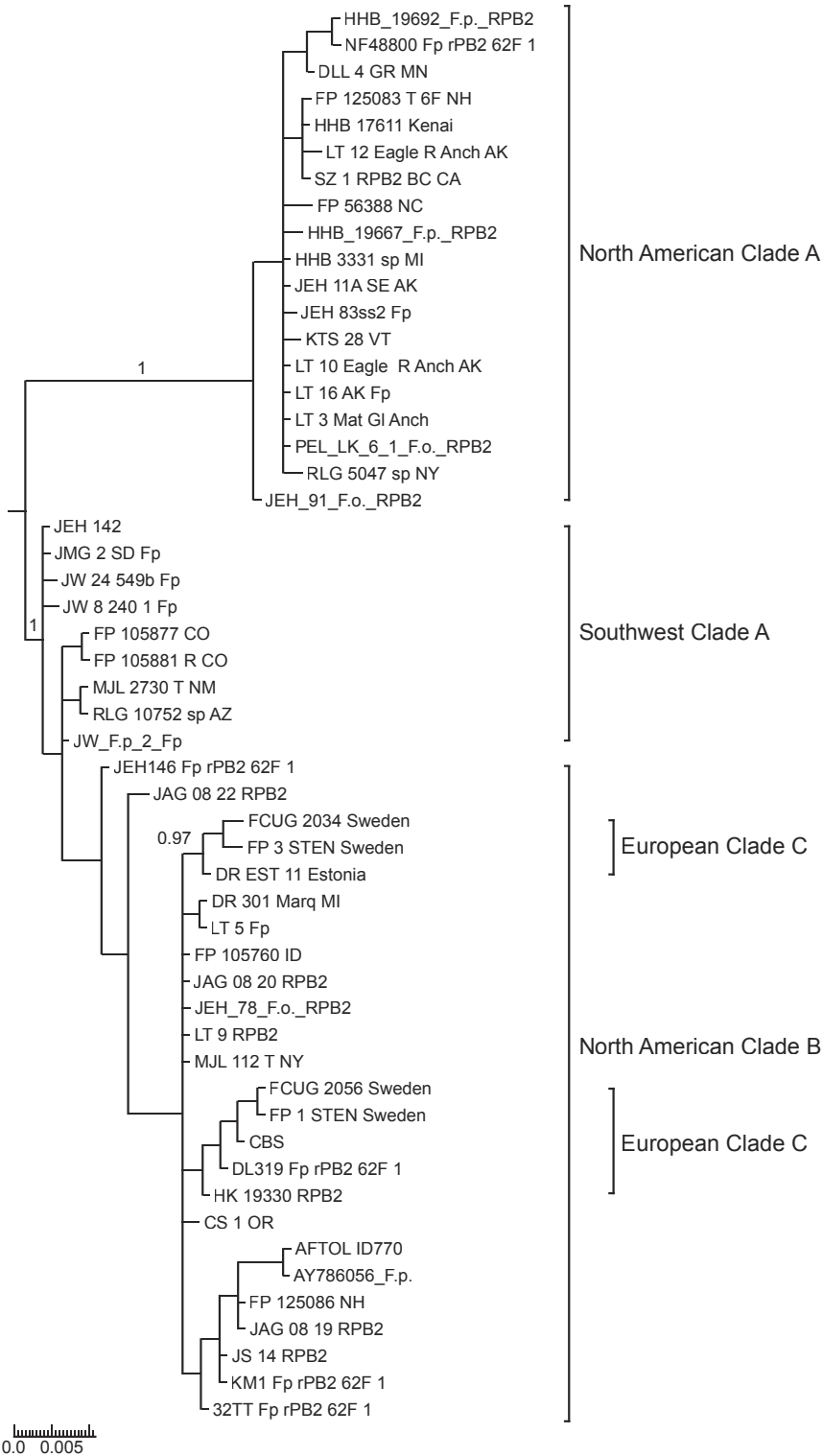


FIG. 1. Bayesian gene tree of RPB2 based on analysis of a 638 base pair, partitioned sequence from a representative sample of 112 specimens of *F. pinicola*. The tree is midpoint rooted and branch support values (PP ≥ 0.95) are shown on individual branches.

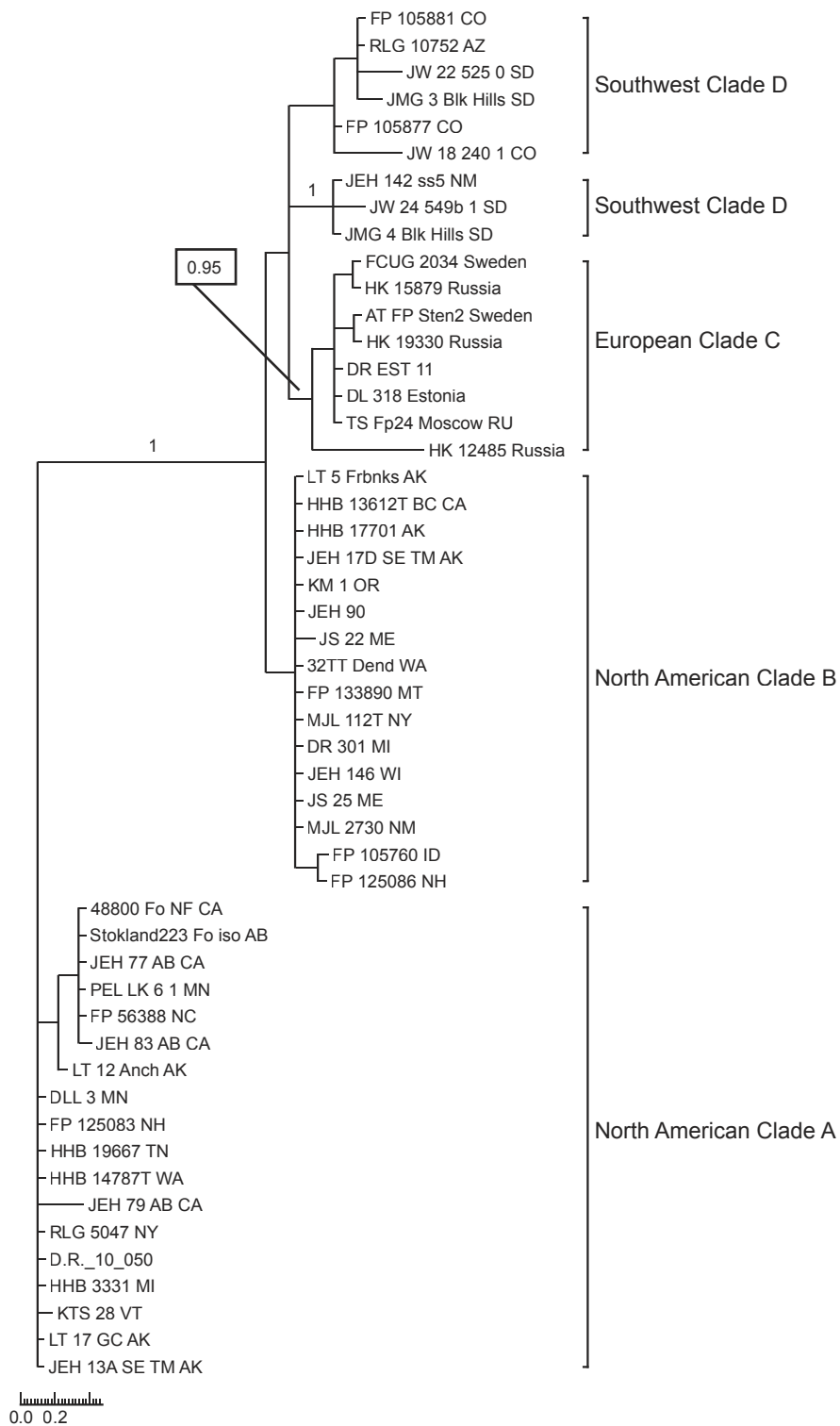


FIG. 2. Bayesian gene tree for EF1A based on analysis of a 557 base pair, partitioned alignment from a representative sample of 132 specimens of *F. pinicola*. The tree is midpoint rooted and branch support values (PP ≥ 0.95) are shown on individual branches.

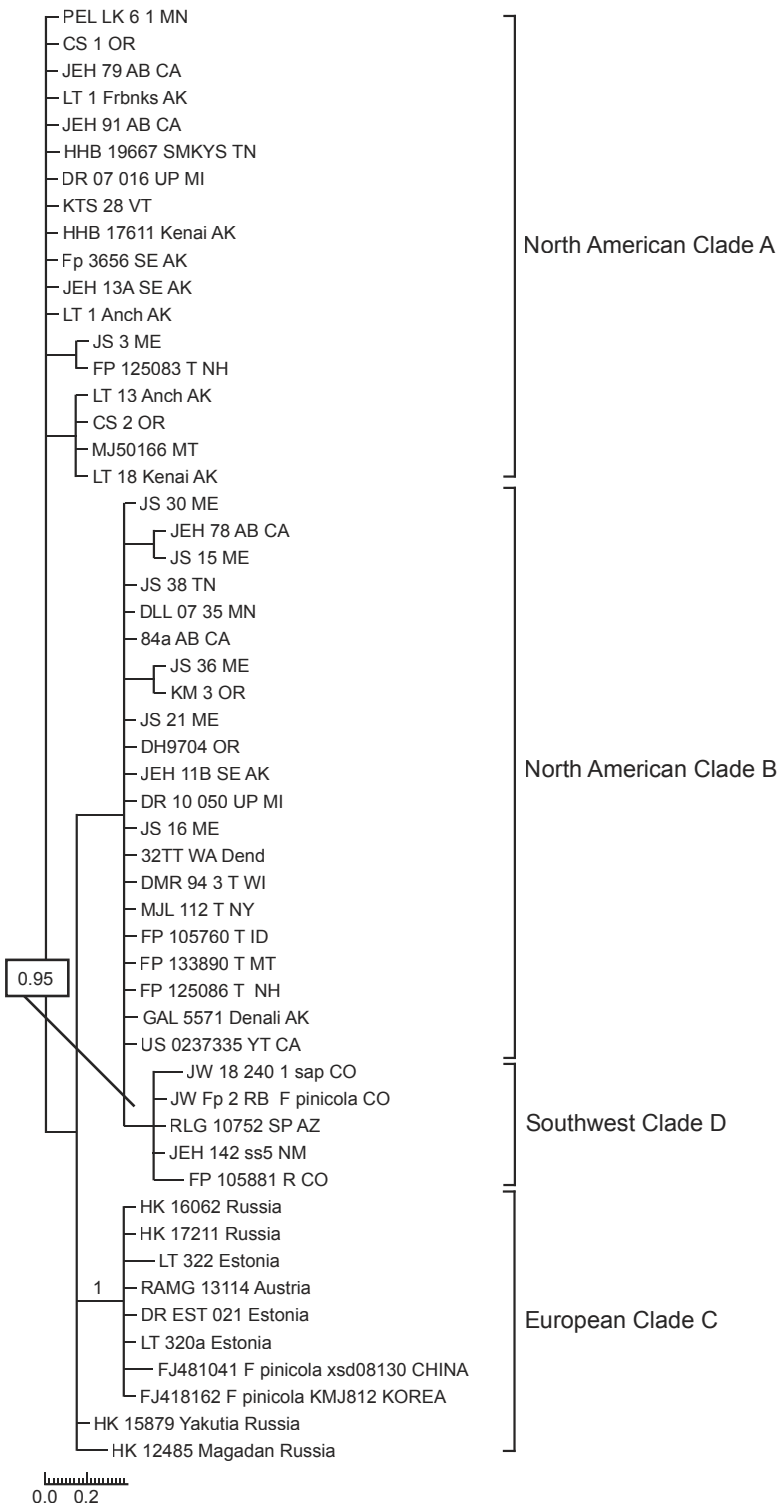


FIG. 3. Bayesian gene tree for ITS based on analysis of a 544 base pair, partitioned alignment from a representative sample of 161 specimens of *F. pinicola*. The tree is midpoint rooted and branch support values (PP \geq 0.95) are shown on individual branches.

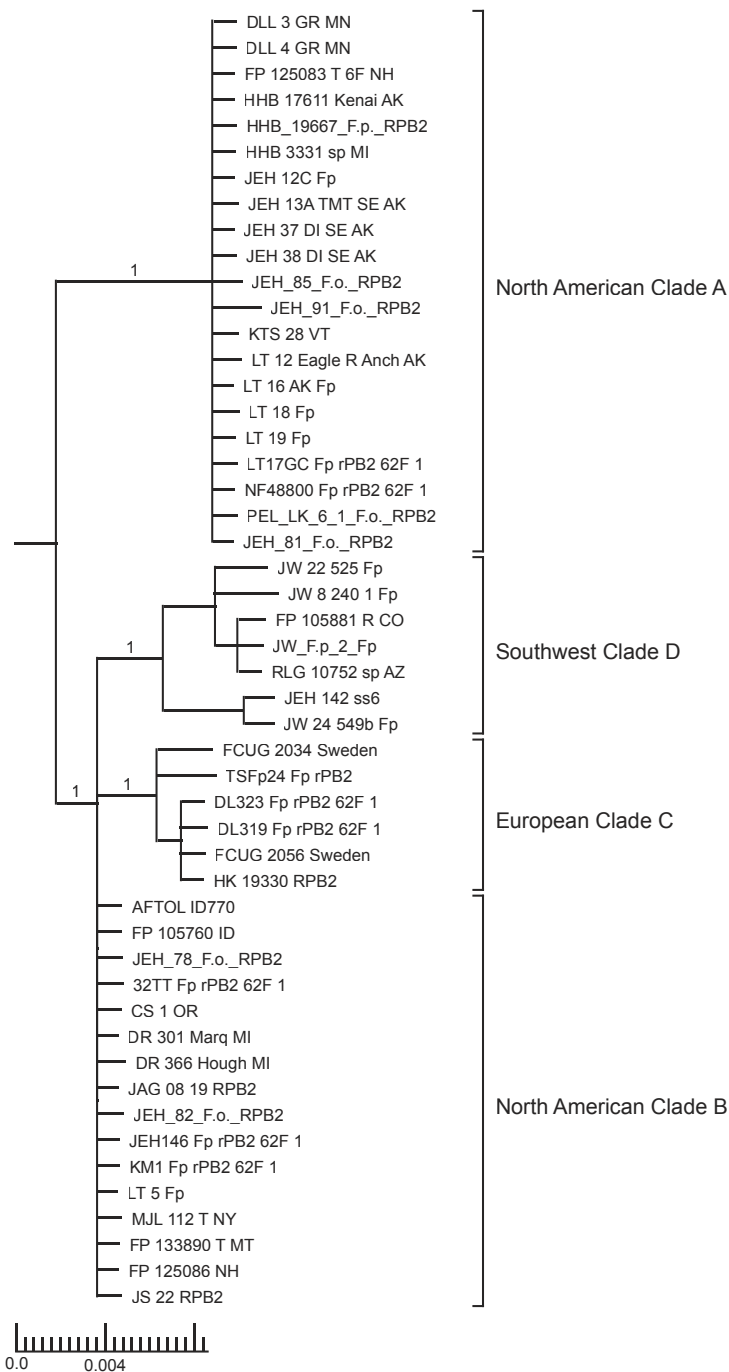


FIG. 4. Bayesian coalescent species tree based on analysis of a 1739 base pair, partitioned sequence of RPB2, EF1A and ITS from a representative sample of 70 specimens of *F. pinicola*. The tree is midpoint rooted and branch support values ($PP \geq 0.95$) are shown on individual branches

into sister species created a guide tree with six species: (((Eur, NA2), (SW1, SW2)), (NA1, NA1a)). The five nodes, which were the same as those in the five-node guide tree again had 100% posterior probability, while the artificial node had posterior probabilities of 23%, 48% and 14% over three runs with different starting trees.

Intra-clade diversity—The number of fixed differences between NAA and NAB, NAA and European Clade C, and NAA and the Southwest clade was significantly higher than the number of fixed differences between NAB and Clade C or NAB and the Southwest Clade (TABLE 3). The number of shared polymorphic sites between NAA and the other clades for each gene was zero, with the exception of RPB2 in the comparison of NAA and Eur. Pairwise nucleotide divergence values (K , D_{xy} , and D_a) were also higher between NAA and the other clades than for comparisons among the other clades. The highest number of shared mutations and the lowest values of pairwise nucleotide divergence occurred between Clades NAB and C. Haplotype diversity for *F. pinicola* (TABLE 4) was higher in Clade C and the Southwest clade (average Clade C H_d 0.728; average Southwest clade H_d 0.732) than that for either NAA or NAB (0.498 and 0.397 respectively). The values for Watterson's θ (θ_w /site) and average pairwise nucleotide diversity (π), were low and similar in each clade. Haplotypes were not shared between the clades.

Table 3. Nucleotide divergence among clades in the *Fomitopsis pinicola* species complex

Clade and locus	Number of polymorphic sites	Number of fixed differences	Polymorphic in Pop 1 but monomorphic in Pop 2	Polymorphic in Pop 2 but monomorphic in Pop 1	Shared mutations	<i>K</i>	<i>Dxy</i>	<i>Da</i>
North America Clade A vs. North America Clade B								
<i>RPB2</i>	60	14	28	20	0	12.189	0.03836	0.03211
<i>EF1-α</i>	23	16	5	2	0	7.866	0.0343	0.03352
ITS	7	0	4	3	0	1.437	0.00547	0.00488
Mean						7.164	0.026	0.0235
North America Clade A vs. European Clade C								
<i>RPB2</i>	59	19	24	14	4	10.669	0.04248	0.03572
<i>EF1-α</i>	29	15	5	9	0	6.834	0.03892	0.03516
ITS	10	1	4	5	0	1.255	0.00598	0.00413
Mean						6.253	0.02913	0.025
North America Clade A vs. Southwest Clade D								
<i>RPB2</i>	46	16	28	4	0	8.652	0.03514	0.03085
<i>EF1-α</i>	28	16	5	8	0	6.442	0.03971	0.03552
ITS	12	4	4	4	0	1.74	0.00956	0.00794
Mean						5.611	0.02814	0.02477
North America Clade B vs. European Clade C								
<i>RPB2</i>	32	0	13	12	7	5.024	0.00892	0.00109
<i>EF1-α</i>	10	0	0	9	1	2.937	0.00964	0.00619
ITS	6	0	1	3	2	1.783	0.00574	0.00376
Mean						3.248	0.0081	0.00368
North America Clade B vs. Southwest Clade D								
<i>RPB2</i>	25	0	19	5	1	6.229	0.01449	0.00907
<i>EF1-α</i>	15	4	0	10	1	3.92	0.01407	0.00987
ITS	8	1	3	4	0	1.298	0.0041	0.00235
Mean						3.816	0.01089	0.0071
European Clade C vs. Southwest Clade D								
<i>RPB2</i>	32	7	19	5	1	8.607	0.01993	0.01361
<i>EF1-α</i>	21	0	9	11	1	5.609	0.01264	0.00504
ITS	9	0	5	4	0	2.623	0.00666	0.00365
Mean						5.613	0.01308	0.00743

K, average number of nucleotide differences between populations; *Dxy*, nucleotide divergence; *Da*, number of net nucleotide substitutions per site between populations.

TABLE 4. Nucleotide polymorphism of clades in the *Fomitopsis pinicola* species complex

Clade	Locus	<i>n</i>	<i>h</i>	<i>S</i>	<i>Hd</i>	θ_w /site	π
North American Clade A							
	<i>RPB2</i>	64	34	26	0.928	0.00903	0.0054
	<i>EF1-α</i>	64	5	5	0.333	0.00209	0.00105
	ITS	64	5	4	0.234	0.00157	0.00046
	Mean				0.498	0.004	0.002
North American Clade B							
	<i>RPB2</i>	30	17	20	0.933	0.00809	0.00689
	<i>EF1-α</i>	30	2	2	0.129	0.00092	0.00047
	ITS	30	2	3	0.129	0.00141	0.00072
	Mean				0.397	0.003	0.003
European Clade C							
	<i>RPB2</i>	16	9	20	0.892	0.00945	0.00878
	<i>EF1-α</i>	16	6	10	0.867	0.00542	0.00659
	ITS	16	3	5	0.425	0.00282	0.00322
	Mean				0.728	0.006	0.006
Southwest Clade D							
	<i>RPB2</i>	14	4	6	0.659	0.00296	0.00386
	<i>EF1-α</i>	14	4	12	0.747	0.00681	0.00857
	ITS	14	5	4	0.791	0.00234	0.00278
	Mean				0.732	0.004	0.005

n is the number of phased haploid sequences; *h* is the number of haplotypes; *S* is the number of segregating sites; *Hd* is the haplotype diversity θ_w is Watterson's theta; π is the average pairwise nucleotide diversity.

DISCUSSION

Clade support—Results of three independent MrBayes analyses of the ITS, EF1A and RPB2 genes indicated the presence of 4-5 clades (populations) within an *F. pinicola* species complex. Some of these clades had high support in the gene trees (FIGS. 1-3). A coalescent species analysis (FIG. 4) supported four clades (NAA, NAB, SW and European Clade C) and these four clades were also supported when implementing Bayesian species delimitation with multilocus sequence data in BPP. Analysis of sequence polymorphism in DnaSP (TABLES 3, 4) supported the conclusions of the phylogenetic and coalescent analyses.

Species concepts—The search for a functional definition for a species that can be applied to all fields of biology has been elusive. Mayden (1997) divides species concepts into two categories, theoretical and operational, with operational concepts regarded as those that are effective in identifying species. Furthering this idea, de Queiroz (2007) separates the discussion into a conceptual problem and a methodological problem, developing a unified species concept, with species defined as separately evolving lineages. Though widely advocated by evolutionary biologists, the idea of species as separately evolving lineages has not been universally accepted (Hausdorf 2011, Baum 2009) and use of the methods of the biological species concept (BSC), the morphological species concept (MSC) and the phylogenetic species concept (PSC), either under the heading of species concepts, species recognition (Taylor et al. 2000) or as secondary defining properties (secondary species criteria) (de Queiroz 2007) will likely continue to be necessary, particularly as applied to the practical problem of recognizing and separating species. The BSC, MSC

and PSC each specify criteria for recognizing species (Taylor et al. 2000). These three concepts are pertinent to addressing species delimitation in sexually reproducing fungi, such as *F. pinicola*.

Biological species comprise groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1940, Mayr 1942). Mayr emphasized that reproductive isolation is key to the definition (Mayr 1942), and Dobzhansky (1937) mentioned the development of reproductive isolation as an important evolutionary step in the formation of new species. Reproductive isolation in sexually reproducing fungi can occur at multiple stages during mating tests, due to reproductive isolation mechanisms. In fungi, these isolating mechanisms can result in failure of anastomosis, failure of plasmogamy, failure to exchange mating type nuclei between compatible cells and failure of karyogamy. Such compatibility barriers in decay fungi often take the visible form of lines of demarcation; barriers of melanized hyphae separating incompatible strains or species. In sexually reproducing basidiomycete fungi such as *F. pinicola*, crosses between single spore isolates can be checked for the formation of clamp connections, an indication that the isolates are not reproductively isolated but belong to the same potential gene pool. The BSC provides a practical means for delineating species genetically and has been the most influential concept in population and evolutionary genetics and conservation biology (Frankham 2002).

In contrast to the BSC, the MSC is not defined by reproduction and can be applied equally to sexual and asexual organisms, which broadens its utility across the Fungi.

Morphologic (taxonomic) species are identified as populations differing statistically in

one or more characteristics (Blackwelder 1967, Cronquist 1978, Raup 1978). These characteristics by definition must be genetically fixed within a population and distinct to the population. Although the morphological species concept cannot be counted on to diagnose evolutionarily meaningful species in fungi (Taylor et al. 2000), the MSC is fundamental to publishing formal species descriptions of fungi and has served as a common standard in deposition of fungal specimens in herbaria for future study.

Morphological descriptions of *F. pinicola* in Europe (Ryvarden and Gilbertson 1993) and North America (Gilbertson and Ryvarden 1986) encompass the variation within characters, especially the broad range in pileus color, found within the species.

A phylogenetic species is the smallest diagnosable cluster of individual organisms within which there is a clear pattern of ancestry and descent (Cracraft 1983). Classification based on a phylogenetic species concept can yield information about evolutionary relationships and the distribution of genetic variation whereas classification based on the biological species concept would lack such information (Hibbett and Donoghue 1996). Among allopatric sibling species, many traits might diverge before the ancestral ability to mate would be lost, particularly since there could be no selection against it (Taylor et al. 2000), therefore the biological species concept may tend to lump genetically isolated groups into one biological species (Taylor et al. 2000). Although all three of these species concepts have recognition criteria, the PSC may be the most informative regarding species delimitation in fungi, because as populations within a species diverge, fixed differences in gene sequences are often detected before changes in mating behavior or morphology are evident (Taylor et al. 2000). Taylor distinguished between the theoretical

and operational species concepts (Mayden 1997), and used the term “species recognition” for the operational ones. Combining the concordance of more than one gene genealogy within the PSC, he developed the operational concept of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al. 2000). Under this principle, areas of concordance between gene trees, due to fixation of alleles caused by genetic drift (reciprocal monophyly), designate phylogenetic species and areas of discord determine species boundaries. The GCPSR has been widely used to delimit fungal species (Dettman et al. 2003, O’Donnell et al. 2004, Peterson and Horn 2009), and may make a better appraisal of true species diversity, since very little of biological significance happens at the moment of genealogical speciation (Hudson and Coyne 2002). Yet, genealogical concordance is a conservative method (Sites and Crandall 1997, Knowles and Carstens 2007, Leavitt et al. 2012) and other methods, such as coalescent theory, may be more sensitive to detecting speciation than genealogical concordance.

Population genetic inference, coalescent theory—Coalescent theory is a model of population genetics that models genetic drift backward through time, tracing polymorphism in a gene back to the most recent common ancestral gene. This process models divergence between gene lineages from the present, when the genes are sampled, back to the time when the genes diverged (Degnan and Salter 2005). At this point all lineages of the gene have coalesced and the resulting product is a gene genealogy. Phylogenetic methods map the evolutionary history of species. Where phylogenetic methods estimate gene trees that represent the pattern of species descent, the coalescent process models the evolution of the gene trees; the random genealogical process that has

given rise to each tree (Rosenberg and Nordborg 2002). Phylogenetic methods generally do not accommodate gene flow or recombination when delimiting species, as this will cause discordance among the gene trees. By contrast, recombination between loci allows them to be interpreted as independent evolutionary replicates, providing statistical benefits important to the evolutionary inference in coalescence (Rosenberg and Nordborg 2002). Although principles of the BSC and PSR were also taken into consideration, our study centered on coalescent theory, using a Bayesian hierarchical model (Liu and Pearl 2007) to estimate the phylogeny of the species complex in *F. pinicola*.

Population genetic inference, Bayesian species delimitation—Coalescent theory is becoming increasingly important in phylogenetics and speciation research (Fujita et al. 2012), yet there is some opposition to delimiting and naming species without corresponding informative diagnostic characters (Bauer et al. 2011, Schipani 2011), which could cause an artificial increase in the number of taxa, merely due to the change in species definition (Agapow et al. 2004) and could also make the identification of unknown specimens difficult. While character-based descriptions are important to describing a new species, at times morphological differentiation lags behind genetic isolation of a species (Taylor et al. 2000) and character states may overlap in the interim, therefore phylogenetic species may not have fixed morphological differences or fixed DNA differences across loci (Fujita and Leaché 2011). Bayesian species delimitation (Yang and Rannala 2010) applied to closely related species accommodates variation among gene trees. The key to successful application of the Bayesian species delimitation is developing an accurate guide tree to minimize inference errors (Yang and Rannala

2010). Various methods of developing a guide tree are possible including concatenating sequence data, combining gene trees or using morphological data (Yang and Rannala 2010). The coalescent species tree used as the guide tree in this study had posterior probabilities > 0.95 for each node delimiting species, and the nodes were fully supported during BPP analysis.

Population genetic inference, population genetics—The addition of population genetic data to a study delimiting species is powerful because it permits us to quantify the genetic polymorphism within and among populations and putative species. Comparison of the number polymorphic sites and number of fixed differences between NAA and the other clades support the high posterior probability values assigned to nodes in the coalescent species tree. The number of fixed differences and shared polymorphisms (TABLE 3) confirm the relatively close relationship between NAB and the European Clade C seen in the gene trees.

Interpreting the F. pinicola complex—Our phylogenetic results suggest that *Fomitopsis pinicola* sensu lato is a species complex comprised of four well-supported phylogenetic species, three in North America and one in Europe. These findings agree with the earlier findings of Irene Mounce (Mounce and McCrae 1938), who performed crosses with single spore isolates of *F. pinicola* and discovered three intersterility groups; two in North America and one in Europe. However, they declined to designate two species in North America due to the compatibility of their Group A with their European isolates and partial incompatibility of their Group B with their European isolates. Mounce isolate 1264 (Mounce and Macrae 1938) is available from the Centraalbureau voor

Schimmelcultures Fungal Biodiversity Centre (CBS 221.39), and Genbank sequences for both RPB2 and EF1a were included in this study and are represented in North American clade B. This isolate links North American clade B with Mounce Group A, and since each study found two clades of overlapping distribution in North America, it is likely that North American clade A from our study corresponds to Group B from their earlier study. We suspect that the Southwest clade uncovered in our study was not sampled or studied by Mounce. Mounce and McCrae tested only two isolates from the geographic range covered by the Southwest clade: one isolate from South Dakota and one isolate from Arizona. Their pairings placed these isolates into Mounce Group A and Group B respectively, and the results of single spore mating tests of these two isolates were similar to other members of their respective groups. Our results suggest that the Southwest clade is sympatric with NAA and NAB in this region, so it is reasonable to suppose that Mounce's isolates from this region belonged to the latter clades.

Significance of host species—Members of North American clade A and B were found on both hardwoods and softwoods. Similarly, Mounce group A and group B each included isolates from hardwoods and softwoods, requiring mating tests to segregate them. The long held hypothesis that fruiting bodies found on species of *Populus*, exhibiting white or grey coloration and lacking the red belt, a character typically associated with *F. pinicola sensu lato*, indicates a variety or even a separate species, as *F. marginatus*, was not supported by phylogenetic evidence in our study. Specimens spanning the range of color types common to *F. pinicola sensu lato* (Gilbertson and Ryvardeen 1986) were found on

both hardwoods and softwoods, and were represented in both North American clade A and B and the Southwest clade.

Evaluation of reinforcement—Reinforcement refers to the enhancement of prezygotic isolation between populations in response to selection against interspecific mating (Servedio and Noor 2003). Sibling species in allopatry likely would not face selection pressure to diverge reproductively, and development of reproductive isolating mechanisms could occur gradually through mutation and genetic drift; requiring only geographic isolation and time (Turelli et al 2001). Therefore, under reinforcement, reproductive isolation should be stronger between sympatric than allopatric sibling species. Reproductive isolation was found to be significantly stronger in sympatry than in allopatry for Homobasidiomycota (Le Gac and Giraud 2008), suggesting the presence of strong selective pressure for the evolution of premating isolation mechanisms.

Presence of premating isolating mechanisms may be what Mounce and McCrae (1938) observed in two groups of *F. pinicola* in North America that were almost completely incompatible with each other, with overlapping geographical distributions similar to clades NAA and NAB uncovered in this study. This could suggest that these North American groups have existed in sympatry for an extended period, and have developed extensive prezygotic isolation barriers between them due to selective pressure. When North American isolates were crossed with those from Europe, from whom they have presumably been isolated from for an extended period of time, the result of only partial incompatibility suggests that they may have been undergoing allopatric speciation and that under the slower pace of genetic drift and mutation, have developed fewer isolating

mechanisms between them. It has been suggested that only species with a strong premating isolation due to sexual partner recognition can coexist in sympatry (Le Gac and Giraud 2008),

Taxonomic implications—Our data suggest that the European Clade C, which has a geographic range confined to Eurasia, is *F. pinicola* (Sw) P. Karst. North America is home to three phylogenetic species designated in this investigation as NAA, NAB and the Southwest Clade. North American Clade A numbers among its members *F. ochracea* (Ryvarden and Stokland 2008), represented by two collections in our study, the isotype (Stokland-223) and another specimen collected by Ryvarden (48800). Based on previous single spore culture studies of Mounce and Macrae (1938) and applying phylogenetic and population-genetic species concepts, we propose that *F. pinicola sensu stricto* is restricted to Europe, that North American Clade A is *F. ochracea*, and that two new species are represented by North American Clade B and the Southwest Clade, which remain to be named. This study contains insufficient data to determine the validity of the subclade within the Southwest Clade in the coalescent species tree. More data should be collected from specimens in this geographic region to help answer this question. Additional morphological and anatomical work is needed to uncover characters that are effective in separating these four species in the *F. pinicola* complex.

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